

CHAPTER 5

GETTING RECOMBINANT PLASMIDS IN BACTERIA

INTRODUCTION

Inserting a gene into a plasmid vector is an important first step in the gene cloning process. However, if the ultimate goal is to produce a large amount of a particular protein, what is your next step? The plasmid must replicate to make sure that there are many copies of the gene, and the gene must be *expressed*—and both activities can only occur inside a cell. Therefore, your next step in the gene cloning process is to put the recombinant plasmid into *E. coli* bacteria through a process that is called *transformation*, because it changes the DNA content of the bacteria. In this chapter, you will carry out the transformation of *E. coli* bacteria using the recombinant plasmid that contains the *rfp* gene.

CHAPTER 5 GOALS

By the end of this chapter, you will be able to do the following:

- Describe the role of transformation in the gene cloning process
- Explain the purpose of each control in the transformation experiment
- Explain how the information encoded in a gene is expressed as a trait

WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about plasmid uptake and gene expression in bacteria.

1. Do you think that bacterial uptake of a plasmid from the environment is a common event? Why or why not?
2. What are the steps involved in transcription and translation of a gene?
3. What is the relationship among genes, proteins, and traits (or observable characteristics)?
4. What do bacteria and humans have in common that makes it possible for a human gene to be expressed in bacteria?

TRANSFORMING BACTERIA WITH RECOMBINANT PLASMIDS

A plasmid is an ideal vector for carrying DNA sequences from one organism to another. The plasmid is equipped with (1) a promoter that enables gene transcription, (2) a sequence for the initiation of DNA replication, and (3) an antibiotic resistance gene. The plasmid can be taken up by bacteria where it replicates, and its genes are expressed using the bacterial cellular machinery. If a gene of interest has been inserted into the vector, the bacteria produces the product encoded by that gene.

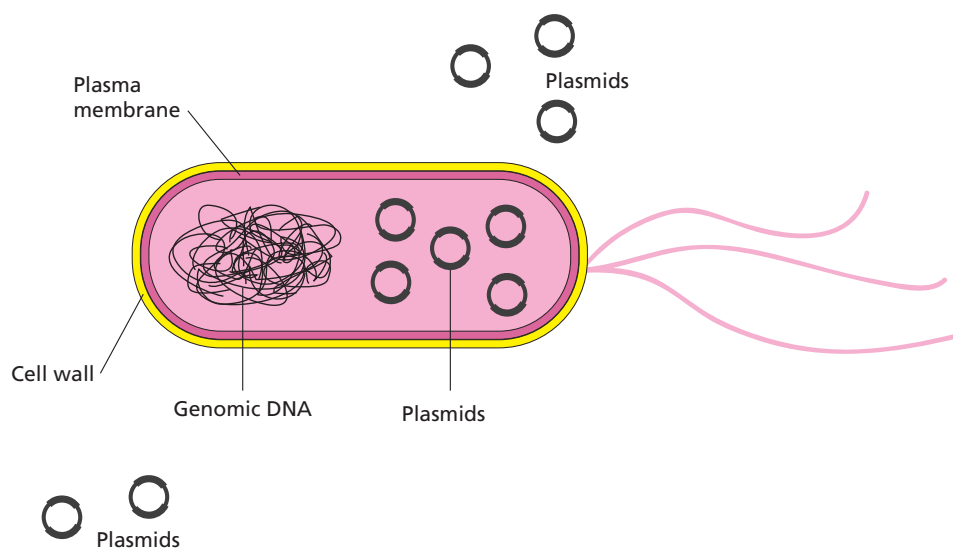


CONSIDER: Once a gene has been inserted into a vector, what do you think is required to make the product encoded by the inserted gene?

BACTERIA TRANSFORMATION

Once a recombinant plasmid is made that contains a gene of interest, such as insulin, the plasmid can enter bacterial cells by a process called *transformation*. Figure 5.1 illustrates transformation.

Figure 5.1: Bacterial transformation



The uptake of DNA from the environment of a bacterial cell occurs with a very low efficiency in nature. *E. coli* bacteria have complex plasma membranes that separate the external environment from the internal environment of the cell and carefully regulate which substances can enter and exit the cell. In addition, the cell wall is negatively charged and repels negatively charged DNA molecules.

CONSIDER: Why is it important that the membranes of *E. coli* bacteria carefully regulate which substances can enter and exit the cell?



In order to increase the efficiency of DNA uptake, bacteria are treated in two ways. First, the *E. coli* bacteria are placed in a solution that contains positive calcium ions, which neutralize the negative charge on the cells' outer membranes, enabling DNA molecules to cross the plasma membranes and enter the cell. Next, the bacteria are subjected to a heat shock, a sudden increase in temperature, which causes the pressure outside the cell to increase. This pressure difference enables the plasmid DNA to enter the bacterial cell from the outside.

Cells treated with calcium and heat are considered *competent* to take up DNA more efficiently, but even with this treatment only about 1 in 10,000 bacterial cells takes up a plasmid in its environment. So how can the bacteria that have taken up the recombinant plasmid be identified? Recall that an important component of a recombinant plasmid is a gene for antibiotic resistance. If you place bacterial cells in the presence of the antibiotic, only those cells that have the recombinant plasmid will grow.

DID YOU KNOW?

Natural Uptake of Plasmids

Some strains of bacteria naturally exchange plasmids, and those plasmids can provide a gene that gives a selective advantage, such as antibiotic resistance, to the cell. One mechanism for efficient transfer of DNA between bacterial species is *bacterial conjugation*, in which a plasmid is shared between two bacterial cells that are in contact. The other mechanism is transformation, in which bacteria take up DNA, including plasmids, directly from the external environment. In nature, the usual source of this DNA is cells that have died and released their contents into the environment.

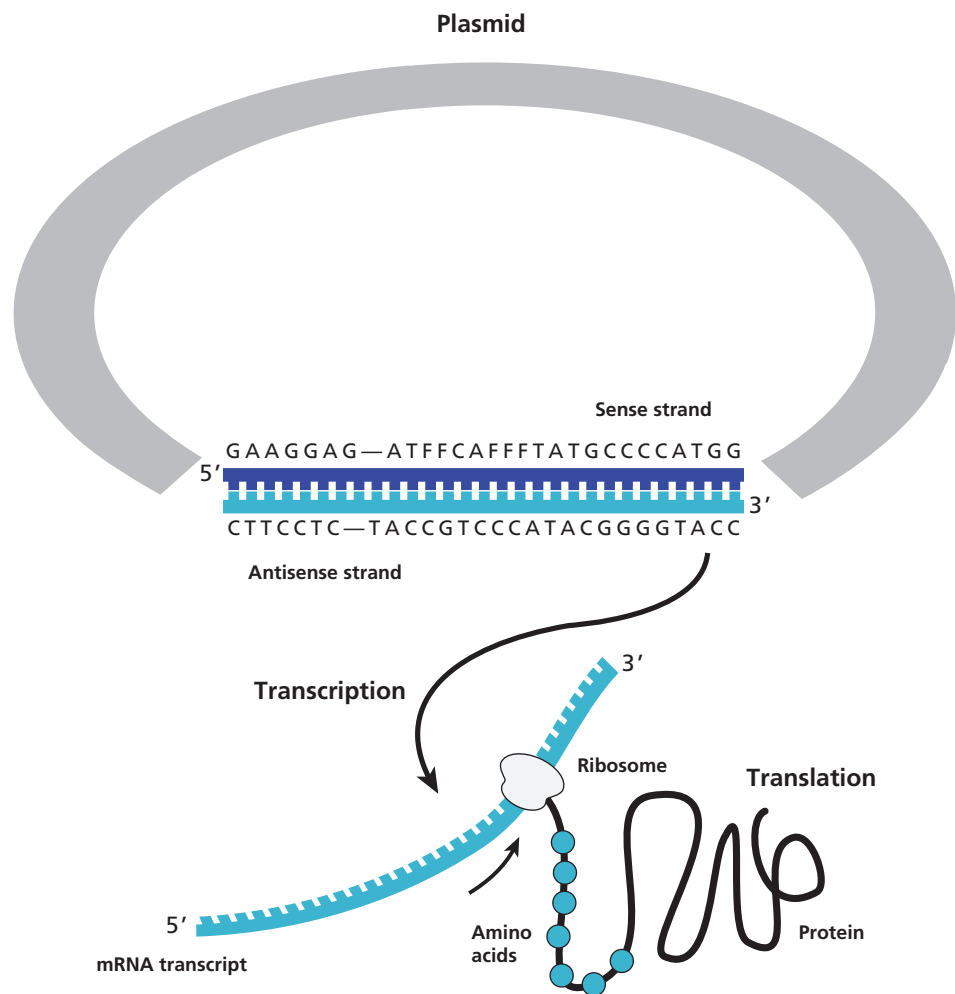
While plasmids can be advantageous for bacteria cells, they are not always advantageous for people. For example, vancomycin was the only effective antibiotic against *Staphylococcus aureus* infections. Resistance to vancomycin is associated with the *VanA* gene, which is carried on a plasmid in other species of bacteria (such as the *Enterococcus faecium* [BM4147] strain). Exchange of this plasmid made vancomycin less effective against staph infections.



FROM PLASMID DNA TO PROTEIN

Once a recombinant plasmid has entered the bacterial cell, DNA polymerase initiates replication at the *ori* site, and the plasmid replicates using the bacterial DNA replication enzymes. These multiple copies of plasmids can now produce the protein of interest, such as insulin, in quantity. In this process, the information encoded in the human DNA is transferred from DNA to protein using the transcription and translation machinery of the cell (see **Figure 5.2**). The protein then alters the observable traits of the organism.

Figure 5.2: Gene expression from a plasmid in the bacterial cell



Genetic engineering is only possible because genes from different organisms can be expressed in bacteria. On Earth, all life is related, and the way that information is encoded in DNA is universal. As you may already know, proteins are made up of smaller subunits called *amino acids*, and a sequence of three nucleotides in DNA code for a single amino acid. These three-nucleotide sequences are called *codons*. For example, the codon TTG codes for the amino acid tryptophan, whereas the codon AAG codes for the amino acid lysine. In many cases, more than one codon can encode the same amino acid. For example, AAA is also a codon for lysine. In addition, there are informational codons, such as the start codon (ATG) and the *stop codon* (TTA), which show where in the DNA sequence the code for the protein begins and ends.

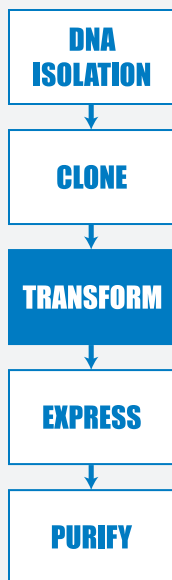
DID YOU KNOW?

Making DNA from RNA

Even though the DNA code is the same in all life forms, the transcription and translation of genes in *eukaryotes* and *prokaryotes* use different enzymes and structures. (Human cells are eukaryotes, and bacterial cells are prokaryotes.) One important difference between these two kinds of cells is that the genes in eukaryotes contain noncoding sequences called *introns*. The RNA polymerase transcribes the gene, producing a large precursor messenger RNA containing both introns and exons, which are the coding sequences. The precursor RNA is then *spliced*, which removes the introns and joins the exons into the mature messenger RNA.

Prokaryotes are unable to carry out the splicing of the introns. To solve this problem, scientists use an enzyme, *reverse transcriptase*, which can copy RNA into DNA, to make complementary DNA (cDNA) from the messenger RNA for a particular protein. The cDNA, which has only the exon sequences, is then inserted into the plasmid vector. The cloned human insulin gene used to make insulin is prepared in this way.





LABORATORY 5: TRANSFORMING BACTERIA WITH THE LIGATION PRODUCTS

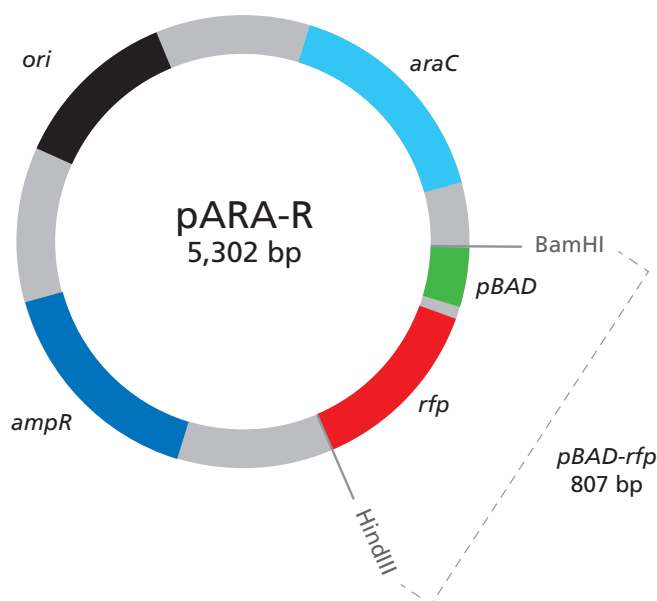
So far in your quest to clone a gene you have produced recombinant plasmids and verified that you made the pARA-R plasmid containing the *rfp* gene that can make the *red fluorescent protein*. In this laboratory you will carry out another step of the gene cloning process, which is to transform *E. coli* bacteria with this plasmid. Using *E. coli* bacteria that have been pretreated with calcium chloride, you will divide the bacteria into two groups: a control group to which no plasmid is added, and a treatment group to which you add the ligation products. After heat-shocking both groups of cells, you will grow them under several different conditions:

- The control group is grown in the presence of Luria Broth (a *medium* that supports bacterial growth).
- The control group is grown in the presence of Luria Broth and the antibiotic ampicillin.
- The treatment group is grown in the presence of Luria Broth.
- The treatment group is grown in the presence of Luria Broth and the antibiotic ampicillin.
- The treatment group is grown in the presence of Luria Broth, ampicillin, and the sugar arabinose.

By examining the growth of bacteria under these conditions, you can verify that your procedure worked, and you can identify the bacteria transformed with the pARA-R plasmid that you created in *Laboratory 3*. How will you know if you are successful? The bacteria will have a new and highly visible trait: It will now produce red fluorescent protein, which makes the cells red or bright pink!

The pARA-R plasmid, which you reviewed in Chapter 3, is shown again in **Figure 5.3**.

Figure 5.3: The pARA-R plasmid



The relevant components of this plasmid are the *rfp* gene, the promoter (*pBAD*), the ampicillin resistance gene (*ampR*), and the arabinose activator protein gene (*araC*). The *ampR* gene confers resistance to the antibiotic ampicillin. The *araC* gene controls the promoter. If arabinose, a simple sugar, is present in the bacteria, the activator protein made by the *araC* gene turns on the promoter, which then binds RNA polymerase, and transcription of the *rfp* gene occurs. Activator proteins are used in some recombinant plasmids to control production of the protein of interest.

HANDOUTS

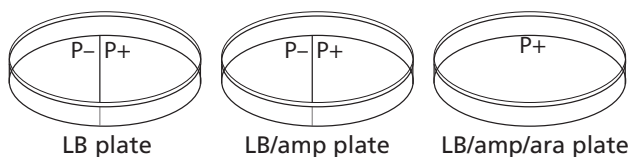
- Bacterial Growth Predictions (RM 5)

BEFORE THE LAB

Discuss the following questions with your group, and be prepared to share your answers with the class.

1. Ampicillin is an antibiotic that kills bacterial cells by disrupting the formation of cell walls. However, the pARA-R plasmid has the ampicillin resistance gene, which produces a protein that breaks down ampicillin. What is the purpose of growing bacteria that have been transformed in the presence of ampicillin?
2. What will happen when bacterial cells that contain the pARA-R plasmid are not given arabinose?

- In the lab, you will add samples of the control group P⁻ and the treatment group P⁺ to plates that contain various combinations of Luria Broth (LB), ampicillin, and the sugar arabinose. The plates will be arranged as follows:



Using the key on **Bacterial Growth Predictions (RM 5)**, show your predictions for the growth you would expect for each combination. Then fill in **Table 1** and **Table 2** in the handout by describing the conclusions that can be drawn if the predicted growth occurs or does not occur.

- Read through the Methods section on pages 93 through 97 and briefly outline the steps, using words and a flowchart.



SAFETY: All appropriate safety precautions and attire required for a science laboratory should be used. Please refer to your teacher's instructions.

SAFETY: Use caution when handling *E. coli* bacteria and use *aseptic technique*.

Aseptic technique is a set of procedures that ensure protection of the lab worker and protection of a bacterial sample, which is necessary for the experiment to be successful. Specifically:

- Do not touch anything that has been or will be in contact with *E. coli* bacteria. Students handling equipment that comes into contact with bacteria should wear gloves.
- Try to avoid spills or contamination of surfaces with anything that has been in contact with *E. coli* bacteria. Immediately inform your teacher if a spill or contamination occurs.
- When you have finished using microfuge tubes, pipette tips, and cell spreaders, place them immediately into the biohazard bag or waste container, as directed by your teacher.
- When directed to do so, place your Petri plates back into the original sleeves and in the biohazard bag.
- Wash your hands well with soap after completing the lab.

MATERIALS

Reagents

- A rack with the following:
 - Microfuge tube of ligated plasmid from *Laboratory 3* (LIG)
 - Microfuge tube of Luria Broth (LB)

- Microfuge tube of 100 μ L of chilled competent *E. coli* cells (CC)

Note: The CC tube must be kept on ice at all times.

- 3 Petri plates with agar:
 - 1 of LB
 - 1 of LB/amp
 - 1 of LB/amp/ara

Equipment and Supplies

- Styrofoam cup of crushed ice

Note: Fill a cup with some of the crushed ice from the container holding the CC tubes before taking a CC tube. You'll need to keep the CC tube on ice at all times.

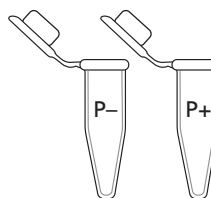
- 2 1.5-mL microfuge tubes
- Permanent marker
- Disposable gloves
- P-20 micropipette
- P-200 micropipette
- Tip box of disposable pipette tips
- Pack of cell spreaders (will be shared among groups)
- 42°C water bath with floating microfuge tube rack (will be shared among all groups)
- Timer or clock (will be shared among all groups)
- Tape (will be shared among all groups)
- 37°C incubator (will be shared among all groups)
- Biohazard bag for materials that come into contact with *E. coli* cells (will be shared among groups)
- Waste container (will be shared among groups)

METHODS

1. Check your rack to make sure that you have the reagents listed. Check that the LIG tube is labeled with your group number and class period.
2. Obtain a CC tube from the ice-filled container, placing it in a Styrofoam cup of ice.

LAB TECHNIQUE: The competent cells in this lab must be kept cold—be sure to pick up microfuge tubes by the upper rim to avoid warming the cells.

3. Label two clean microfuge tubes "P–" and "P+."





4. Place the P– and P+ tubes in the Styrofoam cup of ice with the CC tube.

LAB TECHNIQUE: Bacterial transformation requires sterile techniques. It is essential that these directions be followed precisely.

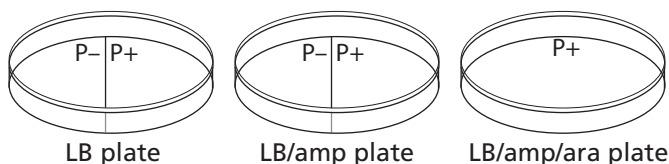
5. Using the large P-200 micropipette, add the competent cells from the CC tube to the P– and P+ tubes:
 - a. Set the P-200 micropipette to 50 μ L.
 - b. Very carefully, re-suspend the bacterial cells in the CC tube by gently pumping the pipette two times in the solution.
 - c. Add 50 μ L of CC to each of the empty chilled tubes (P– and P+), holding each tube at its rim to keep it cold, and return each tube quickly to the ice.

LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each addition.

6. Using the P-20 pipette, add LIG to the tube labeled “P+”:
 - a. Set the P-20 micropipette to 10.0 μ L.
 - b. Hold the chilled P+ tube by the upper rim and add 10.0 μ L of LIG. Mix the solutions by pumping the pipette two times in the liquids, and return the P+ tube to the ice.
7. Keep the P– and P+ tubes on ice for 15 minutes.

NOTE: During the 15-minute interval, share and discuss your answers to question 3 in Before the Lab.

8. While the cells are on ice, prepare your three agar Petri plates—one plate each of LB, LB/amp, and LB/amp/ara:
 - a. Label the bottom of each plate (the part that contains the agar) with your group number and class period. Write small and on the edge of the plate.
 - b. With the plates closed, draw a line on the LB plate and the LB/amp plate that divides each plate in the middle. Label half of each plate “P–” and the other half “P+.” Label the LB/amp/ara plate “P+.” The plates will be arranged as follows:



9. Following the 15-minute incubation on ice, carry the P– and P+ tubes (in the cup of ice) to the 42°C water bath. Place the two tubes in the floating microfuge tube rack in the water bath for exactly 45 seconds.

10. After the 45-second heat shock, immediately place the tubes back on ice and leave them there for at least a minute.
11. Using the large P-200 micropipette, add LB to the P– and P+ tubes:

- a. Set the P-200 micropipette to 150 μL .
- b. Add 150 μL of LB to the P– tube. Cap the tube and gently flick it two or three times to mix.

LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each solution.

- c. Add 150 μL of LB to the P+ tube. Cap the tube and gently flick it two or three times to mix.
12. If time permits, allow the cells in the P– and P+ tubes to incubate at room temperature for 15 minutes.

STOP AND THINK:

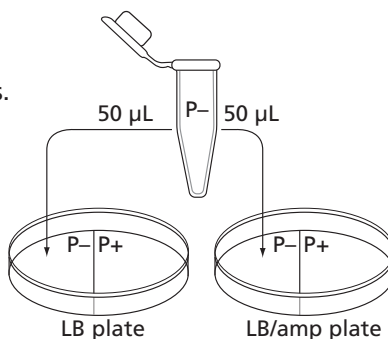
- How is the P+ bacteria culture treated differently from the P– bacteria culture? (A *culture* is an isolated population of cells.) What is the purpose of the P– bacteria culture?
- Why do the cells need time to recover after the heat shock?
- Why are the cells incubated at 37°C?
- You will use aseptic technique in this lab. Why is this important?

13. Add cells from the P– tube onto your LB and LB/amp plates:

- a. Set the P-200 micropipette to 50 μL .

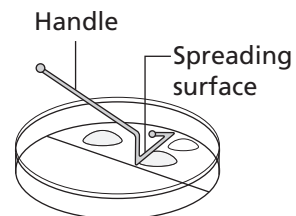
LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each solution.

- b. Gently pump the pipette two or three times in the P– tube to suspend the cells.
- c. Open the lid of the LB plate, like a “clamshell,” and add 50 μL of cells from the P– tube to the section marked “P–.” Close the lid.
- d. Again, gently pump the pipette two or three times in the P– tube to suspend the cells.
- e. Open the lid of the LB/amp plate, like a clamshell, and add 50 μL of cells from the P– tube to the section marked “P–.” Close the lid.



14. Spread the cells from the P⁻ tube on your LB and LB/amp plates:

- Open the package of sterile cell spreaders at the end closest to the spreader handles. Remove only one spreader, and close the package to keep the others sterile.
- Open the lid to the LB plate, like a clamshell, and spread the cells evenly across the entire P⁻ side of the plate by gently moving the spreader across the agar surface. (Keep the cells on the P⁻ side of the plate.) Close the lid.
- Carefully spread the P⁻ cells on the LB/amp plate, using the same spreader and technique.



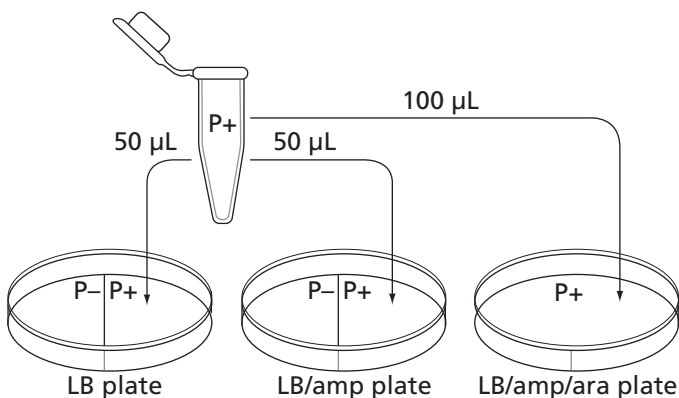
LAB TECHNIQUE: Hold the spreader by the handle and do not allow the bent end to touch any surface, as this will contaminate the spreader. Place the used spreader in the biohazard bag.

15. Add cells from the P⁺ tube to your LB, LB/amp, and LB/amp/ara plates:

- Make sure that the P-200 micropipette is set to 50 μ L.
- Gently pump the pipette two or three times in the P⁺ tube to suspend the cells.

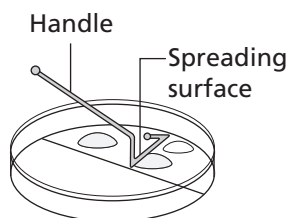
LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each solution.

- Open the lid of the LB plate, like a clamshell, and add 50.0 μ L of cells from the P⁺ tube to the section marked "P⁺." Close the lid.
- Again, gently pump the pipette two or three times in the P⁺ tube to suspend the cells.
- Open the lid of the LB/amp plate, like a clamshell, and add 50.0 μ L of cells from the P⁺ tube to the section marked "P⁺." Close the lid.
- Set the P-200 micropipette to 100 μ L, gently pump the pipette two or three times in the P⁺ tube, and load 100 μ L of the P⁺ cells.
- Open the lid of the LB/amp/ara plate, like a clamshell, and add 100.0 μ L of P⁺ cells to various areas across the surface—not just a single spot. Close the lid.



16. Spread the cells from the P+ tube on your LB, LB/amp, and LB/amp/ara plates:

- a. Open the package of sterile cell spreaders at the end closest to the spreader handles. Remove only one spreader, and close the package to keep the others sterile.
- b. Open the lid to the LB plate, like a clamshell, and evenly spread the cells on the P+ side of the plate (and only on this side) by gently moving the spreader across the agar surface. Close the lid.



LAB TECHNIQUE: Hold the spreader by the handle and do not allow the bent end to touch any surface, as this will contaminate the spreader. Place the used spreader in the biohazard bag.

- c. Carefully spread the P+ cells on the LB/amp plate using the same spreader and technique.
- d. Carefully spread the P+ cells on the LB/amp/ara plate using the same spreader. Then gently rotate the plate beneath the P+ spreader so that the cells can be spread over the entire surface of this plate. Close the lid.

LAB TECHNIQUE: Hold the spreader by the handle and do not allow the bent end to touch any surface, as this will contaminate the spreader. Place the used spreader in the biohazard bag.

17. Allow all three plates to sit right side up for five minutes.
18. Using provided tape, tape all three plates together and label tape with your group number and class period.
19. Place the plates in the 37°C incubator upside down to prevent condensation from dripping onto the gels.
20. Place all microfuge tubes, pipette tips, and cell spreaders in the biohazard bag.
21. Incubate the plates for 24–36 hours at 37°C.
22. Examine the plates and in your notebook record the amount of growth on each half.
23. Discard the Petri plates in the biohazard bag when directed to do so.



CHAPTER 5 QUESTIONS

1. Look at the results of your transformation. Do your actual results match your predicted results? If not, what differences do you see, and what are some explanations for these differences?
2. How many red colonies were present on your LB/amp/ara plate?
3. Why did the red colonies only appear on the LB/amp/ara plate and not the LB/amp plate?
4. Recombinant plasmids are engineered so that they can replicate in the cell independently of the chromosome replication. Why is it important to have multiple copies of a recombinant plasmid within a cell?
5. How is the information encoded in the *rfp* gene expressed as a trait? Be sure to use what you have previously learned about gene expression and the relationship between DNA, RNA, protein, and traits.
6. Why is it possible for bacteria to make a human protein, such as insulin, or a sea anemone protein, such as the red fluorescent dye?
7. The only bacteria that could produce the red fluorescent protein in *Laboratory 5* were bacteria that were transformed with the pARA-R plasmid. Why?



DID YOU KNOW?

Making the Connection Between Genes and Proteins

How were scientists able to show that a gene codes for a protein? In 1941, George Beadle and Edward Tatum carried out an experiment in which they exposed bread mold to UV irradiation, a procedure known to cause *mutations* (changes) in genes. Beadle and Tatum created mutant strains of molds that had lost the ability to synthesize a necessary vitamin. By feeding the precursors of the vitamin one at a time to the mutants, Beadle and Tatum were able to determine that the mutants only lacked a single enzyme catalyzing one reaction.

Beadle and Tatum then investigated whether a single gene caused the loss of the single enzyme by genetic crosses between the mutants and a wild-type strain. After culturing the progeny, they found that half had the same defect as the parent mutant strain and half did not, confirming that a single gene had been mutated. From these results, Beadle and Tatum proposed that genes were responsible for coding the proteins of an organism and that a change in a gene could result in the production of a defective protein, which in turn could affect the traits of that organism. In 1958, Beadle and Tatum received the Nobel Prize for this work.